



## Lipid raft-regulated IGF-1R activation antagonizes TRAIL-induced apoptosis in gastric cancer cells



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### ABSTRACT

Gastric cancer cells are resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the resistance mechanism is not fully understood. In human gastric cancer MGC803 and BGC823 cells, TRAIL induces insulin-like growth factor-1 receptor (IGF-1R) pathway activation. Treatment with IGF-1R inhibitor OSI-906 or small interfering RNAs against IGF-1R, prevents IGF-1R pathway activation and increases TRAIL-induced apoptosis. The TRAIL-induced IGF-1R pathway activation is promoted by IGF-1R translocation into lipid rafts. Moreover, the translocation of IGF-1R into lipid rafts is regulated by Casitas B-lineage lymphoma b (Cbl-b). Taken together, TRAIL-induced IGF-1R activation antagonizes TRAIL-induced apoptosis by Cbl-b-regulated distribution of IGF-1R in lipid rafts.

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### 1. Introduction

Most gastric cancer patients are diagnosed in the advanced stages of the disease. Curative treatment options are limited and mortality is high [1]. To increase therapeutic efficacy, some novel targeted drugs are being clinically tested. As a member of the tumor necrosis factor (TNF) family, TNF-related apoptosis-inducing ligand (TRAIL) is particularly attractive for anti-cancer treatment due to its high specificity and low toxicity [2]. TRAIL binding of death receptor 4 (DR4) and death receptor 5 (DR5), and the recruitment of procaspase-8 through Fas-associated death domain (FADD) for the formation of death-inducing signaling complex (DISC), contributes to its ability to induce apoptosis [3]. Some phase II clinical studies have shown that an agonistic antibody specific for TRAIL receptors is safe and well tolerated in patients with advanced colorectal cancer and non-small cell lung cancer [4,5]. However, it has previously been shown that most gastric cancer cells are insensitive to TRAIL [6,7]. Accordingly, identification of the mechanisms of TRAIL resistance is the key to reversing resistance.

Of the various mechanisms of TRAIL resistance, studies have focused on survival pathway activation. Van et al. demonstrated

that TRAIL-induced phosphorylation of epidermal growth factor receptor (EGFR) resulted in TRAIL resistance in colorectal cancer cells [8]. We recently showed that TRAIL-induced EGFR activation antagonized TRAIL-induced apoptosis in gastric cancer cells, and the inhibition of EGFR activation enhanced TRAIL sensitivity [9]. Whether other survival pathways besides the EGFR pathway are activated to reduce the function of TRAIL is not fully understood. Recently, using protein chips to screen multiple receptor tyrosine kinases, we found that TRAIL also induced insulin-like growth factor-1 receptor (IGF-1R) activation in gastric cancer cells. IGF-1R is expressed in many tumors, and especially in gastrointestinal cancers [10]. Karasic et al. reported that the IGF-1R signaling pathway is highly active in metastatic melanoma cells, and combinatorial treatment with TRAIL and cyclolignan picropodophyllin, a specific inhibitor of IGF-1R kinase activity, enhanced the apoptotic effects on melanoma cells [11]. Moreover, the activation of receptor tyrosine kinase EGFR was regulated by lipid rafts [9,12]. Lipid rafts have also been implicated in the regulation of IGF-1-mediated activation of the PI3K/Akt pathway and survival of mature oligodendrocytes [13]. Disruption of membrane rafts with methyl-beta-cyclodextrin results in inhibition of IGF-1-mediated Akt phosphorylation and altered oligodendrocyte progenitor cell morphology [13]. Since lipid rafts are essential for IGF-1R signaling [14], whether TRAIL-induced IGF-1R activation is involved in TRAIL resistance in gastric cancer cells, and whether TRAIL influences IGF-1R activation through the regulation of lipid rafts warrant further investigation.

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In this study, we show that TRAIL-induced IGF-1R activation antagonizes TRAIL-induced apoptosis. Lipid rafts regulate TRAIL induction of IGF-1R activation by changing IGF-1R distribution.

## 2. Materials and methods

### 2.1. Reagents and antibodies

The dual IGF-1R/IR inhibitor OSI-906 was purchased from SelleckBio (USA). The pan caspase inhibitor zVAD and NF- $\kappa$ B inhibitor PDTC were from Sigma–Aldrich (USA). Recombinant human TRAIL was purchased from Cytolab/Peprotech Asia (USA). Anti-poly (ADP-ribose) polymerase (PARP), anti-caspase-3, anti-caspase-8, anti-IGF-1R, anti-phospho-IGF-1R (Tyr1131), anti-EGFR, anti-phospho-EGFR (Tyr1068), anti-Akt and anti-phospho-Akt (Ser473) antibodies were obtained from Cell Signaling Technology (USA). Anti-ERK, anti-p-ERK 1/2 (Thr 202/Tyr 204), anti-caveolin-1, anti-I $\kappa$ B $\alpha$  and anti-actin antibodies were obtained from Santa Cruz Biotechnology (USA).

### 2.2. Cell cultures

Gastric cancer MGC803 and BGC823 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (China). The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C under an atmosphere of 95% air and 5% CO<sub>2</sub>.

### 2.3. Flow cytometry analysis

Gastric cancer cells were incubated with the indicated concentrations of agents for the indicated times. After fixed with ice-cold 70% ethanol overnight, the samples were incubated with 20  $\mu$ g/ml RNase A at 37 °C and 10  $\mu$ g/ml propidium iodide (PI) for 30 min in the dark. Finally, the samples were evaluated by flow cytometry and the data were analyzed with CellQuest software (Becton Dickinson, San Jose, CA, USA).

### 2.4. Small interfering RNA transfections

IGF-1R small interfering RNA (siRNA) was obtained from Shanghai GeneChem Co. Ltd (China). IGF-1R siRNA was synthesized: 5'-GCATGGTAGCCGAAGATTT-3' (sense). The cells were transiently transfected with IGF-1R siRNA using Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions.

### 2.5. Isolation of lipid rafts

Gastric cancer cells were then solubilized in 150  $\mu$ l of prechilled TXNE buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1% Triton X-100) containing protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin) for 10 min on ice. Subsequently, the cells were scraped off, extracted and moved into 35% Optiprep (Axis-shield, Norway) in polyallomer ultra tubes (Sorvall Instruments, USA) by adding 210  $\mu$ l of 60% Optiprep/0.1% Triton X-100. The cell lysates were covered with 3.5 ml 30% Optiprep in TXNE buffer and 300  $\mu$ l TXNE buffer. After spin in the ultracentrifuge (4 h, 200000 $\times$ g, 4 °C) (Sorvall/Kendro, USA), six fractions were collected from the top. The proteins in fractions 1–2 were taken as the lipid raft fractions.

### 2.6. Western blot analysis

Gastric cancer cells were solubilized in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris–Cl pH 7.4, 150 mM NaCl, 10 mM EDTA,

100 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 2  $\mu$ g/ml aprotinin). Cell lysate proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane (Immoblin-P, Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in TBST buffer (10 mM Tris–Cl pH 7.4, 150 mM NaCl, 0.1% Tween 20) at room temperature for 1 h and incubated overnight at 4 °C with the indicated primary antibodies. After the appropriate secondary antibodies were added for 30–45 min at room temperature, the proteins were detected with enhanced chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate; Pierce, USA) and visualized with the Electrophoresis Gel Imaging Analysis System (DNR Bio-Imaging Systems, Israel).

### 2.7. Reverse-transcription-polymerase chain reaction (RT-PCR)

Gastric cancer cells pellets were washed twice with ice-cold PBS and total RNA extracted with the RNeasy mini kit (Qiagen, Carlsbad, CA, USA) as described by the manufacturer. RT-PCR was performed with primer pairs for IGF-1: forward (5'-CA TTG CTC TCA ACATCTCCC-3') and reverse (5'-ACGAAGTGAAGAGCATCCAC-3'). PCR conditions were 94 °C for 5 min; 33 cycles of 94 °C for 30 s, 59 °C for 45 s, 72 °C for 30 s; one cycle of 72 °C for 10 min. RT-PCR was performed with primer pairs for actin as a control: forward (5'-GTGGGGCGCCCCAGGCACCA-3') and reverse (5'-CT CC TT A ATGTCACGCACGATTTC-3'). PCR conditions were 95 °C for 5 min; 35 cycles of 95 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s; one cycle of 72 °C for 10 min. The amplified products were then separated on 1.5% agarose gels, stained with ethidium bromide and visualized under UV illumination.

### 2.8. Enzyme-linked immunoassay (ELISA)

IGF-1 secretion in the supernatant of MGC803 cells were measured using Human IGF-1 ELISA Kit (CUSABIO Life science, China) following the manufacturer's instructions. We used the professional soft "Curve Expert 1.4" to make a standard curve. According to the fitting curve, enter the standard OD values, get the actual contents of IGF-1.

### 2.9. Statistical analysis

Data were confirmed in three independent experiments and were expressed as the mean  $\pm$  standard deviation (S.D.). Differences between groups were compared using Student's *t*-test. SPSS 20.0 computer software was used for statistical analysis and *P* < 0.05 was considered statistically significant.

## 3. Results

### 3.1. TRAIL induced IGF-1R and downstream Akt and ERK activation in gastric cancer cells

We previously demonstrated that gastric cancer MGC803, BGC823 and SGC7901 cells were resistant to TRAIL [15]. To identify whether TRAIL resistance in gastric cancer cells is associated with the activation of the IGF-1R signaling pathway, IGF-1R and downstream Akt and ERK activation were detected after TRAIL treatment. TRAIL was used at 100 ng/ml in all experiments [6]. Treatment with TRAIL induced the phosphorylation of IGF-1R, Akt and ERK in a temporal manner. In MGC803 cells, the phosphorylation of IGF-1R, Akt and ERK induced by TRAIL started at 2 h post-treatment, reached a peak at 16 h, and declined at 24 h. In BGC823 cells, the phosphorylation of IGF-1R, Akt and ERK gradu-

ally increased and reached a peak at 24 h (Fig. 1). These data suggest that TRAIL resistance in gastric cancer cells may result from the activation of the IGF-1R pathway induced by TRAIL.

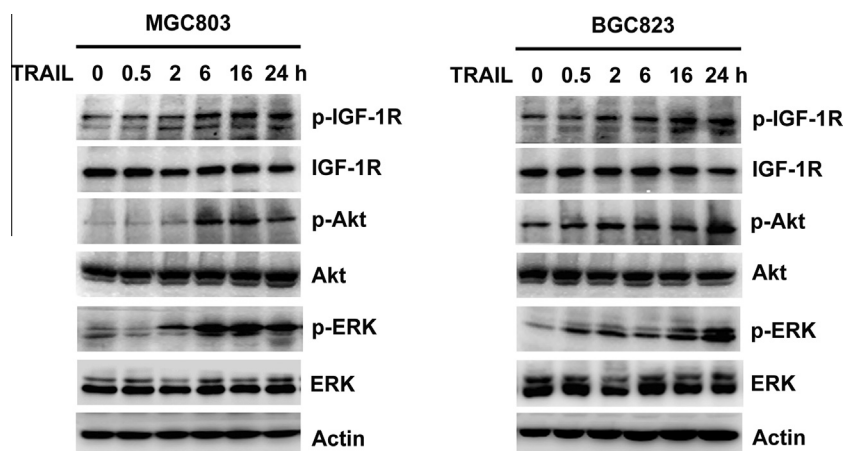
### 3.2. The inhibition of IGF-1R activation increased TRAIL-induced apoptosis in gastric cancer cells

To determine whether inhibition of IGF-1R would sensitize gastric cancer cells to TRAIL, we investigated the effect of OSI-906, a tyrosine kinase dual IGF-1R and insulin receptor (IR) inhibitor, on TRAIL-induced apoptosis in gastric cancer cells. Preincubation with 30  $\mu$ M OSI-906 for 1 h inhibited the TRAIL-induced phosphorylation of IGF-1R, Akt and ERK in MGC803 and BGC823 cells (Fig. 2A). However, the phosphorylation of EGFR induced by TRAIL was not affected by OSI-906 (Fig. 2A). OSI-906 alone had little effect on cell apoptosis after treatment for 24 h (Fig. 2B). Compared with the treatment with TRAIL alone, preincubation with OSI-906 significantly increased the rate of cell apoptosis induced by TRAIL in MGC803 and BGC823 cells ( $20.26 \pm 3.79\%$  vs.  $5.61 \pm 2.56\%$  and  $17.85 \pm 2.97\%$  vs.  $5.92 \pm 2.61\%$ , respectively,  $P < 0.05$ , Fig. 2B). Meanwhile, cleavage of caspase-8, caspase-3 and poly (ADP-ribose) polymerase (PARP) was detected in cells treated with TRAIL and OSI-906 (Fig. 2C). To further delineate the role of IGF-1R activation in TRAIL resistance, MGC803 and BGC823 cells were transiently transfected with IGF-1R siRNA for 48 h. As shown in Fig. 3A, silencing of IGF-1R attenuated TRAIL-induced IGF-1R, Akt and ERK activation. IGF-1R siRNA had no significant effect on apoptosis (Fig. 3B). However, preincubation with IGF-1R siRNA for 48 h followed by TRAIL treatment for 24 h resulted in a significant increase in apoptosis compared with treatment with TRAIL alone in MGC803 and BGC823 cells ( $18.97 \pm 4.07\%$  vs.  $4.97 \pm 1.98\%$  and  $17.28 \pm 3.87\%$  vs.  $5.08 \pm 2.17\%$ , respectively,  $P < 0.05$ , Fig. 3B). Caspase-3 and caspase-8 activation and PARP cleavage were also detected in MGC803 and BGC823 cells (Fig. 3C). These results suggest that inhibition of IGF-1R activation sensitizes gastric cancer cells to TRAIL-induced apoptosis.

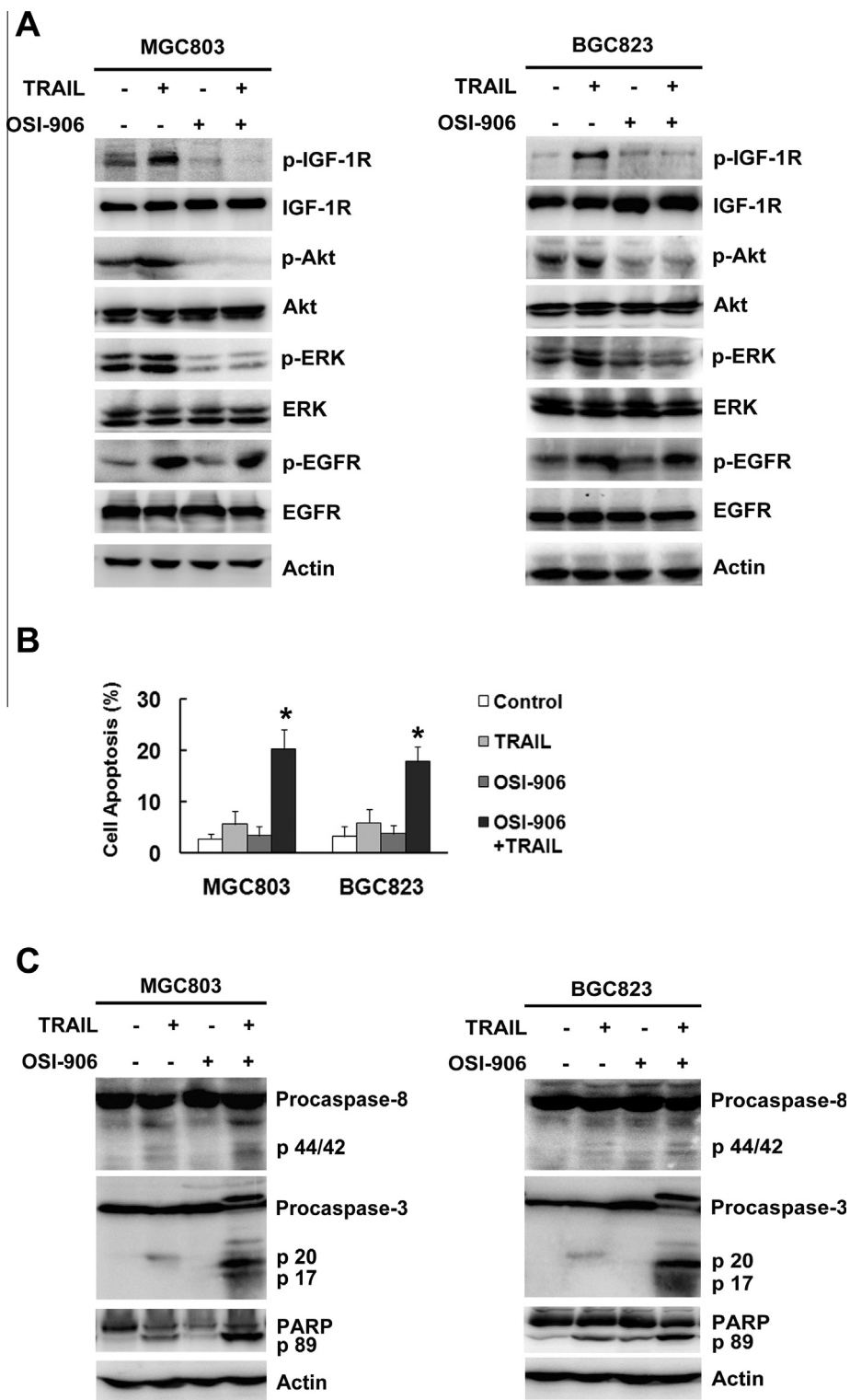
### 3.3. TRAIL induced IGF-1R activation through IGF-1R translocation into lipid rafts in gastric cancer cells

To identify whether endogenous IGF-1 expression and secretion controlled the activation of IGF-1R, we detected IGF-1 mRNA expression and secretion in MGC803 cells after TRAIL treatment. However, IGF-1 mRNA expression was not obviously affected after TRAIL treatment for 2, 6 h (Fig. 4A). Moreover, IGF-1 secretion in the supernatant of MGC803 cells was also not changed after TRAIL treatment

for 2, 6, and 24 h (Fig. 4B). So, TRAIL did not influence IGF-1 expression and secretion, which impossibly further regulated activation of IGF-1R. Our previous study reported that the activation of NF- $\kappa$ B by TRAIL is responsible for resistance to TRAIL in gastric cancer cells [16]. To know whether TRAIL-mediated NF- $\kappa$ B activation controlled IGF-1 and IGF-1R expression levels, we tested IGF-1 mRNA and IGF-1R protein expression after the treatment of TRAIL and PDTC (a NF- $\kappa$ B inhibitor). As shown in Fig. 4C, TRAIL triggered rapid activation of NF- $\kappa$ B, as evidenced by the degradation of I $\kappa$ B $\alpha$  at 6 h, maintained up to 24 h. However, the IGF-1R protein expression levels were not changed. Preincubation with 25  $\mu$ M PDTC for 1 h partially prevented TRAIL-induced the degradation of I $\kappa$ B $\alpha$  at 6 and 24 h, but IGF-1R protein expression was not influenced. In addition, IGF-1 mRNA expression levels were not significantly affected by TRAIL, PDTC or the combination of TRAIL and PDTC for 6 h (Fig. 4D). Thus, TRAIL-mediated NF- $\kappa$ B activation did not regulate IGF-1 and IGF-1R expression levels in MGC803 cells. We recently showed that TRAIL could induce EGFR activation through EGFR translocation into lipid rafts in gastric cancer cells [9]. Since IGF-1R is also located in lipid rafts [17], we investigated whether TRAIL-induced IGF-1R activation was associated with lipid rafts. Membrane lipid rafts were isolated by ultracentrifugation and located using caveolin-1 (fractions 1 and 2, Fig. 5A). We found that TRAIL induced IGF-1R translocation into the lipid raft fractions (1 and 2) in MGC803 and BGC823 cells (Fig. 5A). The percentage of IGF-1R in lipid rafts was enhanced after TRAIL treatment ( $30.83 \pm 4.29\%$  vs.  $10.26 \pm 3.47\%$  and  $19.31 \pm 4.62\%$  vs.  $4.21 \pm 1.98\%$  for MGC803 and BGC823, respectively,  $P < 0.05$ , Fig. 5A). To clarify whether the inhibition of caspase-8 and caspase-3 affect IGF-1R protein traffic in the cell, membrane lipid rafts of MGC803 cells were isolated after the treatment of TRAIL and zVAD (a pan caspase inhibitor). As shown in Fig. 5B, preincubation with 100  $\mu$ M zVAD for 1 h prevented TRAIL-induced cleavage of caspase-8 and caspase-3. However, the distribution of IGF-1R in the lipid raft fractions (1 and 2) was not obviously changed. The percentage of IGF-1R in lipid rafts was respectively  $38.79 \pm 5.07\%$  vs.  $41.08 \pm 3.39\%$  after TRAIL and the combination of TRAIL and zVAD treatment ( $P > 0.05$ ). So, the inhibition of caspase-8 and caspase-3 did not affect IGF-1R distribution in lipid rafts. To further identify whether TRAIL induced IGF-1R activation by lipid rafts, membrane lipid rafts were dissociated by the cholesterol-depleting agent M $\beta$ CD. Preincubation with 2.5 mg/ml M $\beta$ CD for 30 min partially inactivated TRAIL-induced phosphorylation of IGF-1R, Akt and ERK in MGC803 and BGC823 cells (Fig. 5C). The data indicate that TRAIL induces IGF-1R activation by promoting IGF-1R translocation into lipid rafts and the integrity of lipid rafts is necessary for IGF-1R activation in gastric cancer cells.



**Fig. 1.** TRAIL induced the activation of IGF-1R and Akt/ERK in gastric cancer cells. MGC803 and BGC823 cells were incubated with 100 ng/ml TRAIL for 0.5, 2, 6, 16 and 24 h. The phosphorylation of IGF-1R, Akt and ERK was analyzed by Western blot.



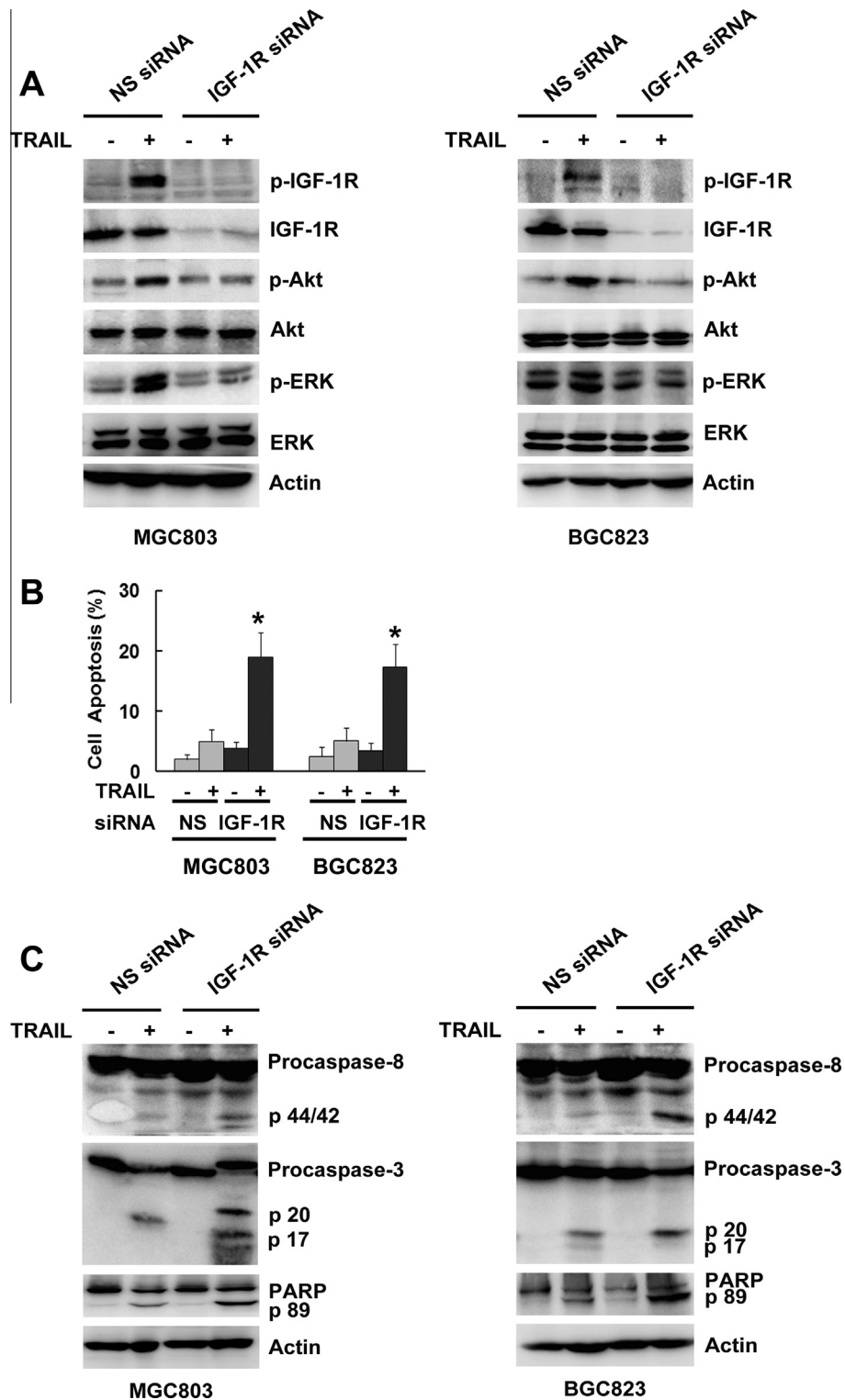
**Fig. 2.** The inhibition of IGF-1R activation increased TRAIL-induced apoptosis in gastric cancer cells. (A) MGC803 and BGC823 cells were preincubated with 30  $\mu$ M IGF-1R inhibitors OSI-906 for 1 h, then treated with 100 ng/ml TRAIL for 16 h. The phosphorylation of IGF-1R, EGFR, Akt and ERK was analyzed by Western blot. (B) MGC803 and BGC823 cells were incubated with 30  $\mu$ M OSI-906 for 1 h, then treated with 100 ng/ml TRAIL for 24 h. Cell apoptosis was quantified with flow cytometry. \*Incubated with OSI-906 and TRAIL vs. with TRAIL alone,  $P < 0.05$ . (C) MGC803 and BGC823 cells were treated under similar conditions to those in Fig. 2B. The expression of caspase-3, caspase-8, and PARP proteins was analyzed by Western blot.

3.4. Knockdown of Cbl-b further promoted IGF-1R translocation into lipid rafts and IGF-1R activation in gastric cancer cells

We previously showed that Cbl-b is a negative regulator of lipid rafts in mast cells [18], and that knockdown of Cbl-b promoted

EGFR translocation into lipid rafts and EGFR activation in gastric cancer cells [9]. To understand the effect of Cbl-b on the distribution of IGF-1R in lipid rafts, shRNA plasmids targeting Cbl-b were transfected into MGC803 cells and stable clones were screened in the presence of G418. The clones with expression levels of Cbl-b

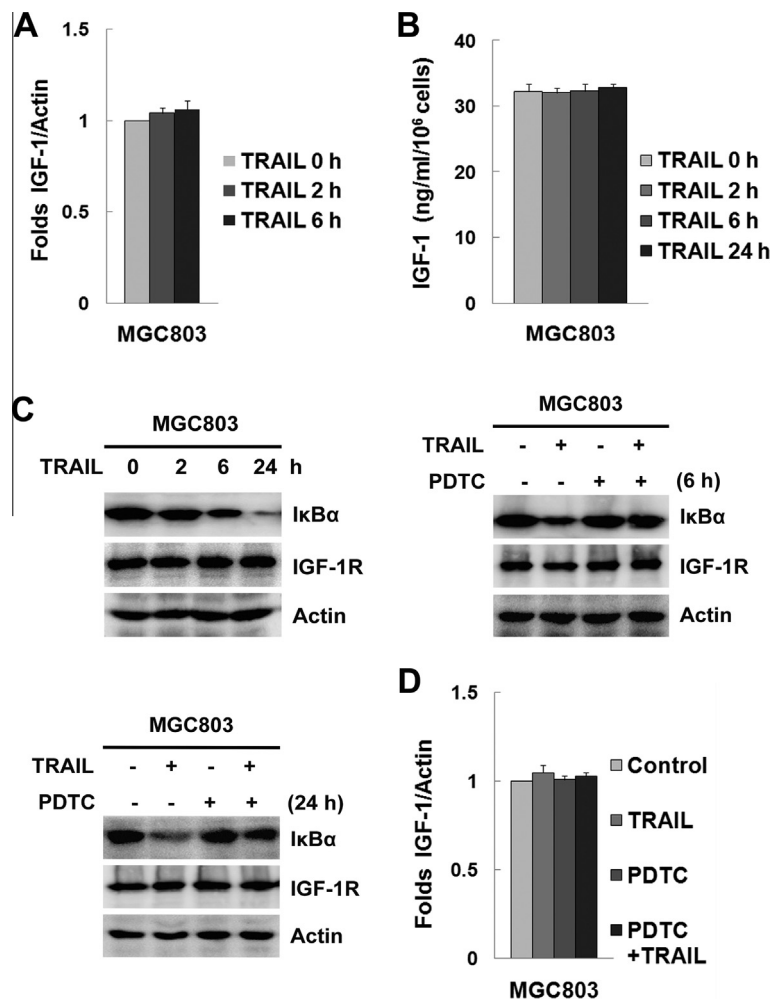




**Fig. 3.** The inhibition of IGF-1R expression sensitized gastric cancer cells to TRAIL. (A) MGC803 and BGC823 cells were transiently transfected with IGF-1R siRNA for 48 h, followed by 100 ng/ml TRAIL for 16 h. The phosphorylation of EGFR, Akt and ERK was analyzed by Western blot. (B) MGC803 and BGC823 cells were transiently transfected with IGF-1R siRNA for 48 h, followed by 100 ng/ml TRAIL for 24 h. Cell apoptosis was quantified by flow cytometry. \*Incubated with TRAIL in IGF-1R siRNA vs. that in non-silenced siRNA (NS siRNA),  $P < 0.05$ . (C) MGC803 and BGC823 cells were treated under similar conditions to those in Fig. 3B. The expression of caspase-3, caspase-8, and PARP proteins was analyzed by Western blot.

less than 10% of the endogenous level were used for subsequent experiments (Fig. 6A and Fig. 7A). In non-silenced controls, Cbl-b translocated into the lipid raft fractions (1 and 2) after TRAIL treatment (Fig. 6A). At the same time, TRAIL induced IGF-1R translocation into the lipid raft fractions (1 and 2, Fig. 6A). The percentage of

IGF-1R in lipid rafts was enhanced after TRAIL treatment ( $38.68 \pm 5.29\%$  vs.  $20.38 \pm 3.46\%$ ,  $P < 0.05$ , Fig. 6A). Importantly, knockdown of Cbl-b further promoted IGF-1R translocation into the lipid raft fractions (1 and 2, Fig. 6A). The percentage of IGF-1R in lipid rafts was further increased after TRAIL treatment

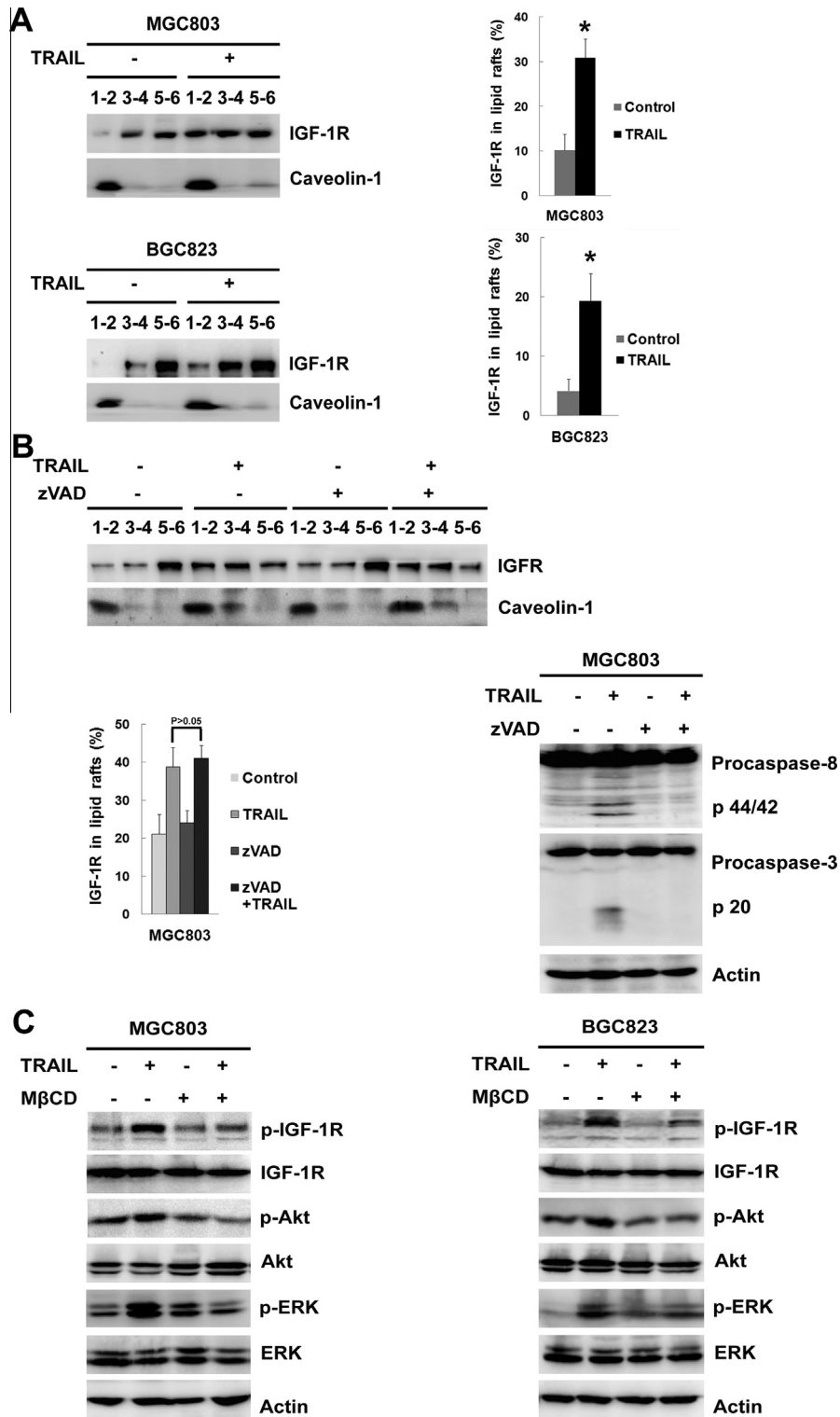


**Fig. 4.** TRAIL did not influence IGF-1 expression and secretion, and NF- $\kappa$ B activation induced by TRAIL did not regulate IGF-1 and IGF-1R expression in gastric cancer cells. (A) MGC803 cells were incubated with 100 ng/ml TRAIL for 2 and 6 h. The expression of mRNA for IGF-1 was analyzed by RT-PCR. Actin was used as an internal control. (B) MGC803 cells were incubated with 100 ng/ml TRAIL for 2, 6 and 24 h. The secretion of IGF-1 in the supernatant was analyzed by ELISA. The data shown are the means  $\pm$  S.D. (ng/ml/10<sup>6</sup> cells). (C) MGC803 cells were incubated with 100 ng/ml TRAIL for 2, 6 and 24 h. The expression of I $\kappa$ B $\alpha$  and IGF-1R proteins was analyzed by Western blot. MGC803 cells were incubated with 100 ng/ml TRAIL, 25  $\mu$ M PDTC or both of them for 6 or 24 h. The expression of I $\kappa$ B $\alpha$  and IGF-1R proteins was analyzed by Western blot. (D) MGC803 cells were incubated with 100 ng/ml TRAIL, 25  $\mu$ M PDTC or both of them for 6 h. The expression of mRNA for IGF-1 was analyzed by RT-PCR. Actin was used as an internal control.

(61.06  $\pm$  6.98% vs. 30.95  $\pm$  4.48%,  $P$  < 0.05, Fig. 6A). Moreover, enhanced phosphorylation of IGF-1R and downstream Akt and ERK was detected in Cbl-b knockdown clones compared with the non-silenced control (Fig. 6B). To further increase the sensitivity of gastric cancer cells to TRAIL, Cbl-b knockdown clones were pre-incubated with 30  $\mu$ M OSI-906 for 1 h followed by TRAIL treatment for 16 h. This led to the partial inhibition of IGF-1R, Akt and ERK phosphorylation (Fig. 7A). Knockdown of Cbl-b enhanced the sensitivity of gastric cancer cells to TRAIL (21.02  $\pm$  3.71% vs. 5.12  $\pm$  2.41%,  $P$  < 0.05, Fig. 7B). In the non-silenced control, preincubation with OSI-906 increased the rate of cell apoptosis induced by TRAIL compared with the treatment with TRAIL alone (17.84  $\pm$  3.28% vs. 5.12  $\pm$  2.41%,  $P$  < 0.05, Fig. 7B). Interestingly, incubation with OSI-906 and TRAIL further increased the rate of cell apoptosis in Cbl-b knockdown clones (37.76  $\pm$  4.86% vs. 21.02  $\pm$  3.71%,  $P$  < 0.05, Fig. 7B). Apoptosis induction was further corroborated by the cleavage of caspase-3, caspase-8 and PARP (Fig. 7C). These findings suggest that TRAIL activates IGF-1R pathway by Cbl-b-regulation of the redistribution of IGF-1R in lipid rafts, and the inhibition of IGF-1R activation further increases TRAIL-induced apoptosis, especially in Cbl-b knockdown clones.

#### 4. Discussion

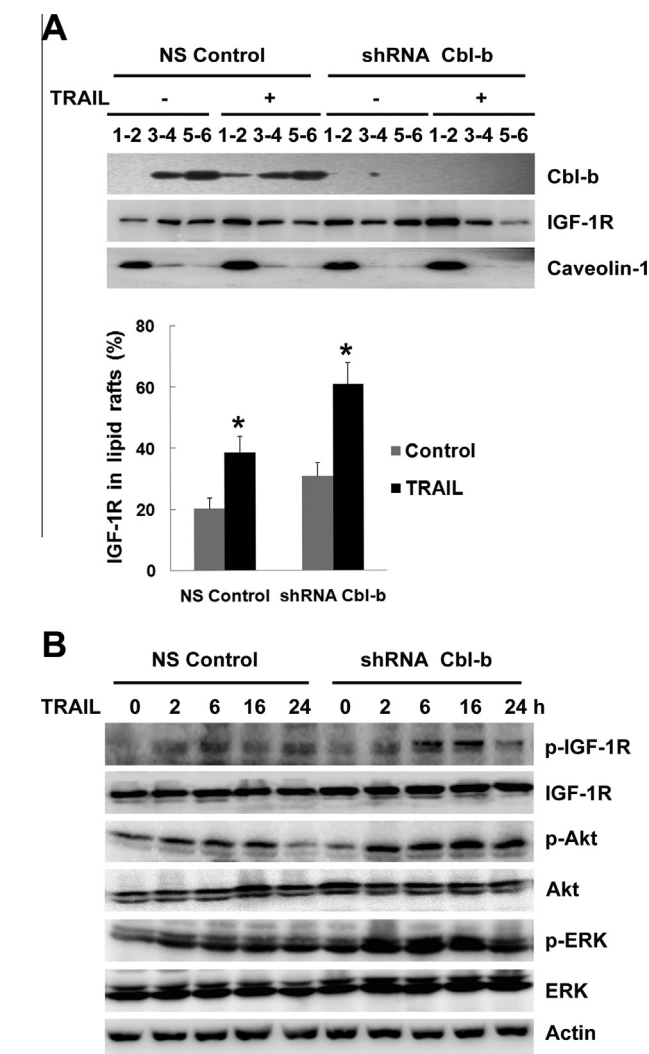
As a promising biological factor, TRAIL is a highly selective molecule which induces apoptosis in malignant cells. However, gastric cancer cells are relatively resistant to TRAIL. Previous studies of the mechanisms of TRAIL apoptosis pathway-associated molecules [19,20]. Recent studies have shown that activation of the survival pathway is another important factor causing TRAIL resistance. Li et al. found that the resistance of nasopharyngeal carcinoma cells to TRAIL was due to the activation of PI3K/Akt signaling pathway [21]. Furthermore, the inhibition of EGFR activation induced by TRAIL could reverse TRAIL resistance in gastric cancer and colorectal cancer cells [8,9]. Moreover, IGF-1R, another kind of receptor tyrosine kinase, activation also protected melanoma cells from TRAIL-induced apoptosis [11]. In this study, TRAIL induced the activation of IGF-1R and downstream Akt/ERK in TRAIL-resistant gastric cancer cells. Treatment with IGF-1R inhibitor OSI-906 or small interfering RNAs against IGF-1R prevented IGF-1R pathway activation, and thus increased TRAIL-induced apoptosis. Hu et al. reported that there was a cross-talk paradigm between IGF-1R



**Fig. 5.** TRAIL activated IGF-1R pathway by promoting IGF-1R translocation into lipid rafts in gastric cancer cells. (A) MGC803 and BGC823 cells were treated with 100 ng/ml TRAIL for 6 h, then were lysed and fractionated by the ultracentrifuge. Locations of lipid rafts (fractions 1–2) were determined using Caveolin-1. The distribution of IGF-1R was analyzed by Western blot. \*Treated with TRAIL vs. untreated with TRAIL,  $P < 0.05$ . (B) MGC803 cells were treated with 100 ng/ml TRAIL, 100  $\mu$ M zVAD or both of them for 6 h, then were lysed and fractionated by the ultracentrifuge. Locations of lipid rafts (fractions 1–2) were determined using Caveolin-1. The distribution of IGF-1R was analyzed by Western blot. Treated with TRAIL vs. treated with TRAIL and zVAD,  $P > 0.05$ . MGC803 cells were treated with 100 ng/ml TRAIL, 100  $\mu$ M zVAD or both of them for 24 h, the expression of caspase-3 and caspase-8 was analyzed by Western blot. (C) MGC803 and BGC823 cells were incubated with 2.5 mg/ml MβCD for 30 min and then treated with 100 ng/ml TRAIL for 16 h. The phosphorylation of EGFR, Akt and ERK was analyzed by Western blot.

and EGFR in colon cancer cells [22]. EGFR activation was downstream of the IGF-1R/PI3K pathway after the exposure to transferin and insulin. Moreover, MAPK/ERK activity was a consequence of EGFR-mediated signaling [22]. To clarify whether IGF-R has an

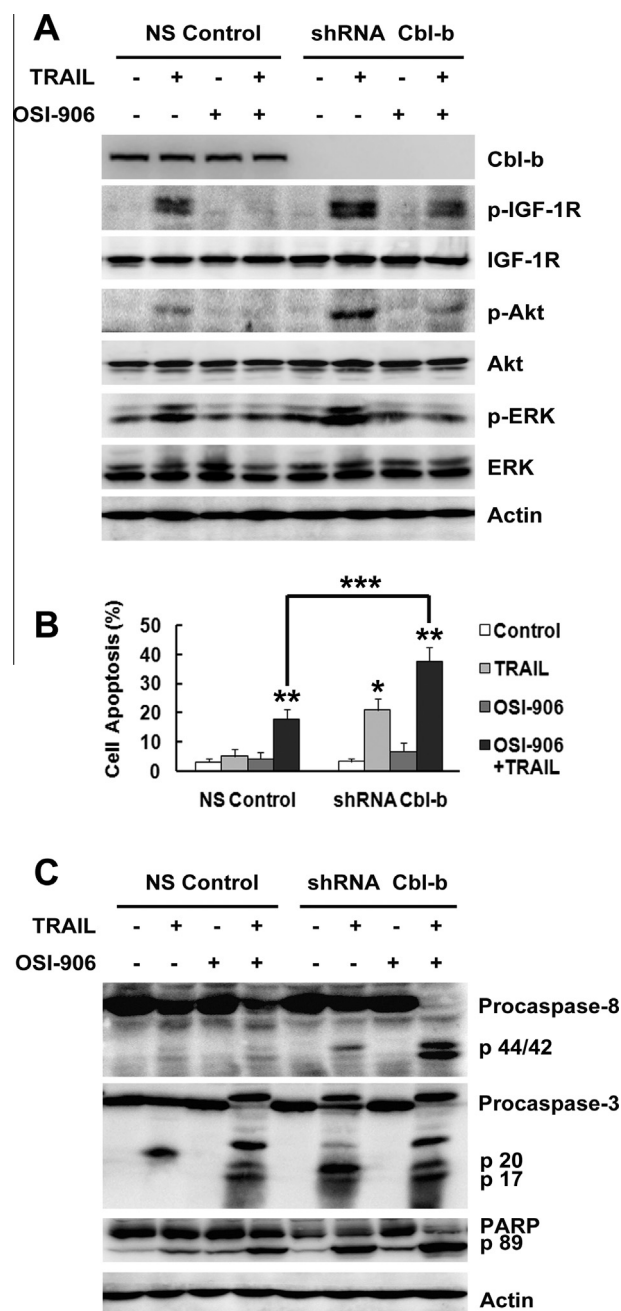
important role in EGFR pathway, we used OSI-906 prevented the activation of IGF-1R and downstream Akt/ERK. However, OSI-906 did not inhibit TRAIL-induced EGFR activation. Thus, EGFR was not downstream of IGF-R, but probably functioned as a parallel



**Fig. 6.** Knockdown of Cbl-b further promoted IGF-1R translocation into lipid rafts and IGF-1R pathway activation in gastric cancer cells. (A) The cells were treated with 100 ng/ml TRAIL for 6 h, then were lysed and fractionated by the ultracentrifuge. The distribution of Cbl-b and IGF-1R was analyzed by Western blot. \*Treated with TRAIL vs. untreated with TRAIL respectively in Cbl-b knockdown cells or in non-silenced controls (NS control),  $P < 0.05$ . (B) The cells were treated with 100 ng/ml TRAIL for 2, 6, 16 and 24 h. The phosphorylation of IGF-1R, Akt and ERK was analyzed by Western blot.

pathway of IGFR in TRAIL-induced apoptosis in gastric cancer cells. TRAIL-induced IGF-1R activation may be another factor causing TRAIL resistance in gastric cancer cells.

IGF ligands regulate tumour growth via autocrine, paracrine and endocrine regulatory mechanism. The most important single components in this process are IGF-1 as well as *IGF-1R* [23]. To identify whether endogenous IGF-1 expression and secretion control the activation of IGF-1R, we detected IGF-1 mRNA expression and secretion in MGC803 cells. In the present study, IGF-1 mRNA expression and IGF-1 secretion in the supernatant of MGC803 cells was not affected after TRAIL treatment, which impossibility further control activation of IGF-1R. In addition, our previous study reported that the activation of NF- $\kappa$ B by TRAIL is responsible for resistance to TRAIL in gastric cancer cells [16]. However, whether TRAIL-mediated NF- $\kappa$ B activation controlled IGF-1 and IGF-1R expression levels is not clear. In the present study, TRAIL triggered rapid activation of NF- $\kappa$ B, as evidenced by the degradation of I $\kappa$ B $\alpha$ . However, IGF-1 mRNA and IGF-1R protein expression were also not affected by TRAIL and NF- $\kappa$ B inhibitor PDTC. Thus, TRAIL-mediated NF- $\kappa$ B activation did not regulate IGF-1 and IGF-1R expression



**Fig. 7.** The inhibition of IGF-1R activation enhanced TRAIL-induced apoptosis, especially in Cbl-b knockdown cells. (A) The cells were preincubated with 30  $\mu$ M IGF-1R inhibitors OSI-906 for 1 h, then treated with 100 ng/ml TRAIL for 16 h. The phosphorylation of IGF-1R, Akt and ERK was analyzed by Western blot. (B) The cells were incubated with 30  $\mu$ M OSI-906 for 1 h, then treated with 100 ng/ml TRAIL for 24 h. Cell apoptosis was quantified with flow cytometry. \*Incubated with TRAIL in Cbl-b knockdown cells vs. that in NS control,  $P < 0.05$ . \*\*Incubated with OSI-906 and TRAIL vs. with TRAIL alone respectively in Cbl-b knockdown cells or NS control,  $P < 0.05$ . \*\*\*Incubated with OSI-906 and TRAIL in Cbl-b knockdown cells vs. that in NS control,  $P < 0.05$ . (C) The cells were treated under similar conditions to those in Fig. 6B. The expression of caspase-3, caspase-8, and PARP proteins was analyzed by Western blot.

levels in MGC803 cells. Receptor tyrosine kinase activation is regulated by many factors. Except for non-receptor tyrosine kinase Src [24], lipid rafts have been shown to provide a platform for triggering receptor tyrosine kinase activation, as seen with EGFR [25]. Similar to EGFR, IGF-1R is also a type of transmembrane receptor protein and located in lipid rafts [26]. Although IGF-1R activation is known to be involved in TRAIL resistance in melanoma cells



[11], how IGF-1R is activated has not been resolved. Here we showed that TRAIL induces IGF-1R translocation into lipid rafts accompanied by IGF-1R pathway activation in MGC803 and BGC823 cells. Although zVAD prevented TRAIL-induced cleavage of caspase-8 and caspase-3, but IGF-1R protein distribution in lipid rafts was not obviously changed. Furthermore, preincubation with the cholesterol-depleting agent M $\beta$ CD partially prevented IGF-1R pathway activation induced by TRAIL. Thus, TRAIL induces IGF-1R activation through lipid rafts in gastric cancer cells. In addition, ubiquitin ligase Cbl-b is a lipid raft negative regulator [27], and knockdown of Cbl-b significantly promoted EGFR translocation into lipid rafts and subsequent EGFR activation [9]. We demonstrated that Cbl-b also regulates IGF-1R distribution in lipid rafts. Knockdown of Cbl-b enhanced IGF-1R translocation into lipid rafts and IGF-1R activation. Therefore, Cbl-b-regulated IGF-1R distribution in lipid rafts is probably an important factor for IGF-1R activation, which results in TRAIL resistance in gastric cancer cells.

In summary, TRAIL-induced IGF-1R activation by Cbl-b-regulated IGF-1R distribution in lipid rafts antagonizes TRAIL-induced apoptosis. Therefore, inhibition of IGF-1R signal triggered in lipid rafts is vital for increasing the sensitivity of gastric cancer cells to TRAIL.

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